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QUINE INTELLECTUAL PROPERTY LAW GROUP, P.C.

By: Evelyn Gomez

Appl. No.	:	10/044,463	Confirmation No. 9878
Applicant	:	Davide R. Grassetti, et al.	
Filed	:	January 10, 2002	
TC/A.U.	:	1617	
Examiner	:	Shengjun Wang	
Docket No.	:	107-000110US	
Customer No.	:	22798	

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APPEAL BRIEF

REAL PARTY IN INTEREST

The real part of interest in the present appeal is Grassetti Family Trust the assignee of the above-referenced application.

RELATED APPEALS AND INTERFERENCES

Appellant, Appellant's Attorney, and the assignee of the above-referenced application are unaware of any appeals or interferences that will directly affect, be directly affected by, or have a bearing on, the Board's decision in the present appeal.

STATUS OF CLAIMS

On March 11, 2009, Appellant appealed from the final rejection of claims 1 to 3, 5 to 16 and 20 to 24. As originally filed, the case included claims 1 to 24. In the Restriction Action of June 15, 2005, Appellant was requested to elect species. Based on the species election of July 3, 2005, the Office withdrew claims 3-4, 7-8, and 13-16 from further

consideration, as being drawn to non-elected species. Claims 4, and 17 to 19 were cancelled in Appellant's Request for Continued Examination (RCE) of August 9, 2006. Accordingly, Appellants believe that claims 1, 2, 5, 6, 10 to 12, and 20 to 24 are under consideration.

STATEMENT OF AMENDMENTS

The claims were not amended in response to the final Office Action mailed November 13, 2008. Accordingly, the appealed claims are the claims as provided in the Amendments of the RCE dated August 9, 2006, as filed in response to the Office Action dated January 14, 2008.

SUMMARY OF CLAIMED SUBJECT MATTER

Appellants' invention provides methods of modulating an immune response by administering effective amounts of thione-forming disulfide (TFD) compounds of certain structure to an identified individual in need, wherein the individual is other than an individual infected with a retrovirus. Support for the claims can be found throughout the specification, e.g., in the Examples section starting at paragraph 129. Specifically, support for identifying an individual in need of immune response modulation and administration of TFDs can be found at paragraphs 33, 43, 108, 111 and in the section entitled "Immunomodulation in Treatment of Diseases and other Ailments" starting at paragraph 113 and in the section entitled "Administration of Thione-Forming Disulfides starting at paragraph 93. Support for The TFD structures of the claims can be found, e.g., at paragraph 67.

The appealed claims are set forth in Appendix A.

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

In the final Office Action dated November 13, 2008 (and continued in the Advisory Action of January 21, 2009) all claims under consideration, including claims 1, 2, 5, 6, 10 to 12, and 20 to 24, were rejected under title 35 § 102(b) based on alleged anticipation by Grassetti et al., U.S. patent 5,662,364.

ARGUMENT

Rejection of claims 1, 2, 5, 6, 10 to 12, and 20 to 24 under 35 U.S.C. §102(b).

Claims 1, 2, 5, 6, 10 to 12, and 20 to 24 were rejected under 35 U.S.C. §102(b) as allegedly anticipated by Grassetti (U.S. 4,378,364), as evidenced by Barber (5,662,896) and Tagawa (Current Pharm. Design 6:681 (2000)). These claims stand or fall together.

The rejections are inappropriate because the Examiner has failed to state a proper case. Moreover, the rejections of these claims are inappropriate because the cited reference does not explicitly or inherently teach at least: 1) methods comprising identifying an individual in need of immune response modulation or 2) administering a thion-forming disulfide (TFD) to an individual in need of immune response modulation. Rejected independent claim 1 is as follows:

> 1. (Previously presented) A method for modulating an immune response comprising:
>
> identifying an individual in need of immune response modulation;
>
> administering to the individual in need of immune response modulation an effective amount of a thione-forming disulfide comprising
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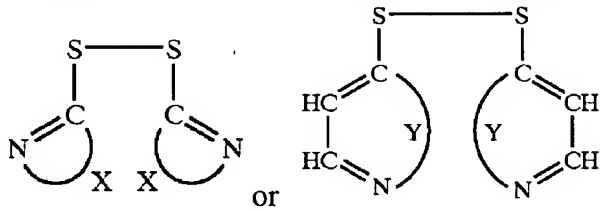
> 
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>
> wherein X and Y represent atoms necessary to form a five-membered or six-membered substituted or unsubstituted heterocyclic ring;
>
> wherein the immune response is selected from the group consisting of: a cellular response, a humoral response and an innate immune response; and,
>
> wherein the individual is other than an individual infected with a retrovirus;
>
> thereby modulating the immune response.

In order for a reference to anticipate an invention, the reference must teach each and every element of the claimed invention. That is, in order for a reference to anticipate an invention, "all limitations of the claim are found in the reference, or 'fully met' by it." *Kalman v. Kimberly-Clark Corp.*, 218 USPQ 781, 789 (Fed. Cir. 1983).

As a preliminary matter, Appellants note that the present rejections are essentially the same rejections for alleged anticipation argued in the Request for Review of

October 18, 2008, and the Decision in the matter was in the favor of Appellants. The continued arguments for rejection are not substantially modified. Appellants again note that Grassetti '364 does not teach all limitations of the claims, and again note that the cited evidence of record actually supports the fact that not all cancer patients are in need of immune response modulation.

Grassetti '364 does not teach at least identifying an individual in need of immune response modulation. This is a limitation in all the rejected claims. At section 4 of the final Office Action of November 13, 2008 (the Action), the Office finds the method step of "'identifying an individual in need of immune response modulation' is [allegedly] inherently met by the method of treating cancer patients ... as all cancer patients are recognized as 'in need of immune response modulation' see abstract in Tagawa and columns 1-2 in Berber et al." Emphasis added. In the Response of December 11, 2008, Appellants refused to take Official Notice of the Examiner's interpretation (according to MPEP 2144.03) of the cited references, and noted that the cited references actually taught that not all cancer patients are in need of immune response modulation.

Appellants have previously shown that the references cited by the Office stand for the principle that not all cancer patients are in need of immune modulation. See, e.g., Response of July 22, 2008, bottom of page 7. For example, the Examiner's Tagawa reference, Figure 1, shows how an unmodulated immune system normally works with, e.g., natural antigen presenting cells (APCs) activating cytotoxic T-lymphocytes (CTLs) to provide a normal CTL-mediated unmodulated immune response against a tumor in vivo. The Examiner's Barber reference, at the cited columns 1 and 2, makes it clear that cancer patients are not necessarily in need of immune modulation. For example, at column 1, line 35, Barber suggests that 30% of patients treated with surgery alone will have no recurrence. The continued allegation that "all cancer patients are recognized as in need of immune response modulation" ignores the facts and remarks on the record regarding the Office's own references. Appellants have repeatedly raised these facts during prosecution, but they have never been specifically addressed on the record by the Office, as required by MPEP 704.14(b) Examiner's Obligation Following Applicant's Reply.

MPEP 2144.03 and controlling case law (e.g., *In re Zurko*, 258 F.3d 1379, 1385, 59 USPQ2d 1693, 1697 (Fed. Cir. 2001); and, *In re Ahlert*, 424 F.2d 1088, 1091, 165

USPQ 418, 420 (CCPA 1970) require the Office to provide reasonable support for allegations of what one of skill would "recognize". Appellants have repeatedly pointed out that the facts in the references are contrary to the rationale in the rejection. Still, the final Office Action and Advisory action repeat the refuted allegations without addressing the facts provided by Appellants in prior Responses. Therefore, the Office fails to state a case because the Examiner has never actually stated a fact-based case and unrebutted facts on the record from Appellants show that not all cancer patients are in need of immune modulation.

Because not all cancer patients are in need of immune response modulation, identification of cancer patients as a group does not identify an individual in need of immune response modulation. Because Grassetti '364 does not teach at least identifying an individual in need of immune response modulation, the claims can not be considered anticipated and the rejections must be withdrawn.

Grassetti '364 does not inherently administer a TFD to any individual in need of immune response modulation. This is a limitation to all the rejected claims. The Office has continued to use a previously discredited inherency argument to allegedly find Grassetti '364 administering a TFD to an individual identified as in need of immune response modulation. At section 7 of the Action, "Grassetti's method [allegedly] would have inevitably [inherently] practiced the claimed method ... even [if] the 'in need thereof' is narrowly interpreted as 30% of patients undergoing chemotherapy." Emphasis added. As a preliminary matter, "inevitably" is in the future and can not be cited as an inherently existing aspect of the reference at the time of filing. Further, such administration was not inevitable.

Controlling case law requires that a rejection based on an inherency argument must present evidence that the missing descriptive matter is necessarily present in the thing described in the reference. See, e.g., See, *Ex parte Levy*, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990); *Continental Can Co. USA v. Monsanto Co.*, 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991); and *In re Oelrich* , 666 F.2d 578, 581 - 82, 212 USPQ 323, 326 (CCPA 1981).

In the Action, at the top of page 5, the Examiner allegedly finds the "administering" limitation in Grassetti '364 allegedly teaching "the employment of the same compound for treatment of the same patients with the same amounts ..." However, this is not the case. For example, Grassetti does not describe identifying an individual in need, does not

necessarily treat the same patients, and does not necessarily treat with the same amounts (e.g., because the clinical end points may be different for the intended appetite enhancement as compared to immune response modulation).

As a preliminary matter, the allegation clearly does not provide facts showing the Grassetti '364 methods would inevitably practice "identification of an individual in need", as discussed above. In fact, the allegation rests on the contrary scenario provided in which many typical patients must be acknowledged as not in need (as evidenced by, e.g., Tagawa and Barber).

Even assuming some cancer patients need immune response modulation, the Action has not alleged that any individual patient in Grassetti '364 was actually in need of immune response modulation. An individual patient in need of immune response modulation is not necessarily described in Grassetti '364, and so does not inherently exist in the reference. The Action refers to "massive treatment of patients" but this aspect is drawn out of thin air and not actually practiced in Grassetti '364. Further, even if Grassetti '364 massively treated patients (and he does not), administration of an effective amount of TFD to an individual in need would not necessarily have taken place.

With regard to section 3 of the Action, traversing pages 3 and 4, the Examiner declines to give weight to the claim aspect of "modulating an immune response". Appellants note that the aspect of modulating an immune response is not only present in the preamble of the claim, but in the body. For example, "modulating the immune response" is present in the last clause of independent claim 1. Therefore, regarding, e.g., independent claim1, the aspect must be given weight.

With regard to section 5 of the Action, concerning *In re Swinehart* and inherent properties of compositions, Appellants direct the Board of Appeals to remarks of Appellants' February 7, 2007, Response, at page 8. For example, *Swinehart* is not on point because the rejected method claims are distinguished over the prior art by more than a mere inherent function of compositions in the prior art. That is, the present claims are not limited only to, e.g., a composition of CPDS but are methods claims with additional limitations.

With regard to section 7 of the Action, concerning paragraph [0100] of the present specification, Appellants direct the Board of Appeals to the unaddressed remarks at pages 5 to 7 of the Response of July 22, 2008. For example, the logic of the rejection does

not require that patients undergoing chemotherapy are in need of immunotherapy just because some patients may have lower than normal numbers of immune cells.

In summary, Grassetti '364 does not explicitly or inherently teach all limitations of the claims. Further, the stated rationale for the rejections is faulty and fails to state a *prima facie* case.

CONCLUSION

Appellants submit that the Examiner's rejection of claims 1, 2, 5, 6, 10 to 12, and 20 to 24 is improper. Withdrawal of this rejection by the Examiner or reversal of this rejection by the Board is respectfully requested.

The Commissioner is authorized to charge the fee under 37 C.F.R. §1.17(c) and any other required fees, or to credit any overpayments, to Deposit Account 50-0893.

If a telephone conference would expedite prosecution of the above-identified application, the Examiner is invited to phone the undersigned at (510) 769-3510.

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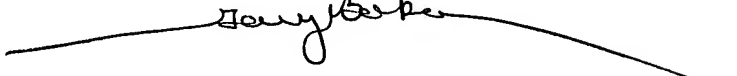
Respectfully submitted,



Gary Baker
Reg. No: 41,595

Attachments:

1) Appendix A – Appealed Claims for 10/044,463;
2) Appendix B - Tagawa (Current Pharm. Design 6:681, 2000);
3) Appendix C - Related Proceedings;
4) A fee transmittal sheet; and,
5) A receipt indication postcard.

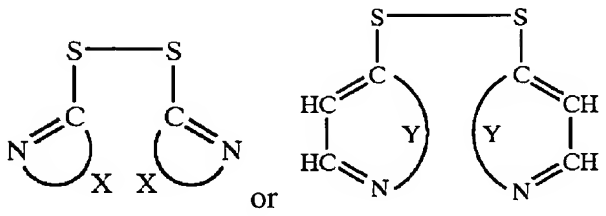
APPENDIX A

APPEALED CLAIMS FOR 10/044,463

1. (Previously presented) A method for modulating an immune response comprising:

identifying an individual in need of immune response modulation;

administering to the individual in need of immune response modulation an effective amount of a thione-forming disulfide comprising



or

wherein X and Y represent atoms necessary to form a five-membered or six-membered substituted or unsubstituted heterocyclic ring;

wherein the immune response is selected from the group consisting of: a cellular response, a humoral response and an innate immune response; and,

wherein the individual is other than an individual infected with a retrovirus;

thereby modulating the immune response.

2. (Original) The method according to claim **1** wherein the immune response is a cellular immune response.

3. (Withdrawn) The method according to claim **2** wherein the cellular immune response is a T cell response and wherein cell populations are increased or lymphoproliferative activity is increased.

4. (Cancelled)

5. (Original) The method according to claim **1** wherein the immune response is an innate immune response.

6. (Original) The method according to claim **5** wherein the innate immune response comprises increasing the natural killer cell population and NK activity.

7. (Withdrawn) The method according to claim **1** wherein the immune response is a humoral immune response.

8. (Withdrawn) The method according to claim **7** wherein the humoral immune response is a decrease in B cell population or B cell response.

9. (Withdrawn) The method according to claim **8** wherein the humoral immune response is an increase or decrease in antibody secretion.

10. (Original) The method according to claim **1** wherein the immune response is biased towards a Th1-type response.

11. (Original) The method according to claim **10** wherein the Th1-type response is an increased cell population of NK cells or T cells.

12. (Original) The method according to claim **10** wherein the Th1-type response is an increased activity in NK cells or T cells.

13. (Withdrawn) The method according to claim **1** wherein the immune response is an increase in cytokine levels.

14. (Withdrawn) The method according to claim **13** wherein the cytokine is selected from the group consisting of IL-2, IFN-.gamma., IFN-.alpha., IFN-.beta., IL-12, TNF-.alpha., and TNF-.beta..

15. (Withdrawn) The method according to claim **1** wherein the immune response is an increase in chemokine levels.

16. (Withdrawn) The method according to claim **15** wherein the chemokine is selected from the group consisting of RANTES, IL-8, MIP-1.alpha., MIP-1.beta., MCP-1, lymphotactin, and eotaxin.

Claims 17 to 19. (Cancelled)

20. (Previously presented) The method according to claim **1** wherein the thione-forming disulfide heterocyclic rings comprise further heteroatoms selected from the group consisting of N, O, and S.

21. (Previously presented) The method according to claim **20** wherein the five- or six-membered heterocyclic ring comprises one or more negatively charged substituents.

22. (Previously presented) The method according to claim **1** wherein one or both of the heterocyclic rings in the thione-forming disulfide comprises a pyridinyl, pyrimidinyl, thiazolyl, or quinolinyl group.

23. (Previously presented) A method of modulating an immune response comprising:

identifying an individual in need of immune response modulation; and,

administering to the individual an effective amount of thione-forming disulfides wherein the compound is selected from the group consisting of: 6,6'-dithiodinicotinic acid

(CPDS), 6,6'-dithiodinicotinic acid diethyl ester, 4-carboxypyrimidine-2-disulfide, diethyl 2,2'-dithiobis-(4-thiazol- e carboxylate), and 2,2'-dithiobis-isonicotinic acid;

wherein the individual is other than an individual infected with a retrovirus; and,

wherein the immune response is selected from the group consisting of: a cellular response, a humoral response and an innate immune response.

24. (Original) The method according to claim **23** wherein the thione-forming disulfides are administered in a pharmaceutically acceptable carrier.

APPENDIX B

EVIDENCE APPENDIX

Tagawa (Current Pharm. Design 6:681, 2000)

Cytokine Therapy for Cancer

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Abstract: Modulation of immune responses by the use of recombinant cytokines or cytokine genes is one of the strategies for cancer therapy. Although host immune responses are complex and many kinds of cells are involved, crucial steps for enhancing anti-tumor responses can be induced by a single or a few cytokines administered. However, cytokines may induce toxic reactions or produce no substantial effects, when the concentration is inappropriate. Administration of recombinant cytokine(s) has advantages in controlling the blood concentration and the biological activity that can be induced by the cytokine. Since cytokines are relatively unstable *in vivo*, cancer patients have to receive a large amount of the recombinant protein to maintain the required blood concentration for biological activity. Administration of the protein is thereby often toxic to the patients. In contrast, secretion of the cytokine from tumor or vehicle cells by gene transfer is another therapeutic maneuver. Previous preclinical studies have shown that cytokines which facilitate type 1 helper T (Th1) cells-mediated immune reactions but not Th2 cells-mediated reactions, when produced in tumors, are effective for anti-tumor responses. Several technical problems to express sufficient amounts of cytokines in appropriate target cells remain unresolved but the potential of cytokine gene therapy is being explored. Cytokine therapy trials also contributes to our present knowledge of how anti-tumor responses can be efectively produced in cancer patients, shedding the light on the generation of tumor-specific immunity in the patients.

INTRODUCTION

Cytokines possess pleiotropic functions and mediate systemic and local biological actions. Host immune defense system is composed of a complexity of cellular and humoral mechanism and a number of cytokines and chemokines are involved in each step. Identification of these cytokine and chemokine genes has enabled us to dissect the complex reactions and to advance our knowledge on how an immune system is operated. A host defense system is influenced by many kinds of cytokine, and systemic and/or local applications of cytokine molecule(s) to the patients of immunological disorders can be one of the possible therapeutic strategies.

Development of tumors can be in part due to a defect of a host immunosurveillance system and an escape mechanism of tumors from host immune responses may play an important role in the progression of tumors. Suppression of the immune system by immunosuppressive agents can increase the frequency of cancer incidence, and fortification of the host defense mechanism may reduce the incidence. Anti-tumor responses have been gauged in experimental animal models. Based on experimental studies, administration of cytokines can possibly drive an immune system from an anergic state to tumor cells into an activated stage. The role of cytokines in immune responses is not unidirectional. They work for suppression or activation of immune responses under a specific condition. To develop cytokine-mediated therapeutic strategies, we have to understand the roles of cytokines in systemic host defense mechanism. Generation of anti-tumor responses requires multi-step pathways, and many phases of cellular and humoral actions contribute to the establishment of systemic immunity.

Cytokine therapy for cancer is based on the understanding of how an immune system works against tumor cells. To produce effective anti-tumor responses, the mechanism of cytokine-mediated immune responses should be clarified. Although numerous kinds of cytokines operate in an immune system, it is not practical to administer many sets of cytokines or cytokine genes into cancer patients. We have to choose a few cytokines or cytokine genes which can activate an

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immune system appropriately and make the system effective for cancer treatment. A number of preclinical and clinical studies are required to testify the assumption that administration of cytokines or cytokine genes is a potential treatment for cancer patients. In this review, I discuss the cytokines that can be used for cancer treatment, their basic immunological background and their possible clinical use.

ANTI-TUMOR RESPONSE INDUCED *IN VIVO*

Various kinds of hematopoietic cells are responsible for successful induction of anti-tumor responses. Cytotoxic T lymphocytes (CTLs) are one of the major cell populations, which destroy tumor cells through at least two distinct pathways, a Fas/Fas ligand (FasL) interaction-induced cell death and a specific proteolytic process that is mediated by perforin-granzymes [1]. In the Fas-mediated pathway, FasL on the surface of CTLs binds to its receptor, Fas , which is expressed on target cells, and the cross-linking of the receptors triggers the cascade system toward programmed cell death. The perforin-granzyme pathway is unique to CTLs and these molecules are stored within granules of CTLs. T cell receptor (TCR)-mediated binding of target cells by CTLs stimulates a Ca^{2+}-dependent degranulation. Subsequently, a pore-forming agent perforin, and serine proteases granzymes are released into the local environment between the target cells and CTLs. This process generates lysis of target cells. The similar cell-mediated apoptosis occurs in the case of target cell killing that is mediated by natural killer (NK) cells.

To develop CTLs that are specific for a certain type of tumor cells demands several procedures such as presentation of putative tumor antigens and expansion of CTL precursor cells. Engagement of TCR on CTLs is also required for target cell killing. On the other hand, NK cells which belong to a cell population operating in the innate immunity, kill tumor cells whose expression of class I antigens of major histocompatibility complex (MHC) is negative or is significantly reduced [2]. NK cells do not kill class I-positive cells such as normal cells. Therefore, CTLs and NK cells have complementary functions in the cytolytic response. While CTLs recognize target cells that express "unfamiliar" peptide(s) in the context of class I antigens, NK cells look for the cells that are devoid of class I molecules.

Recently, a new cell population of T cells, $NK1.1^+T$ cells, has been identified [3,4]. The population expresses restricted αβ TCR and NK1.1 antigen, a member of the family of NKR-P1 NK cell receptors [5]. The TCR is consisted of invariant α chain, Vα14-Jα281 [6], and polyclonal Vβ8, Vβ7 or Vβ2 chain in mice [7]. The similarly restricted TCR complex, invariant Vα24JαQ and a diverse β chain from a Vβ11 gene segment is observed in human [8]. The restricted TCR recognizes the CD1 antigen, a conserved MHC class I molecule, and a particular sort of glycosylceramides embedded with the CD1 molecule [8,9]. Although the immunological significance of $NK1.1^+T$ cells is not well characterized, this cell population has cytolytic activity toward tumor cells [10,11].

The precise mechanism of how NK cells and $NK1.1^+T$ cells kill tumor cells are not clear yet, but that of CTL-mediated cytotoxic activity has been extensively analyzed. It includes antigen presentation by professional antigen presenting cells (APC), expansion of CTL precursors, recruitment of CTLs into tumors and recognition of tumors. Presentation of putative tumor antigen(s) to helper T cells is an initial step to generate anti-tumor responses. Tumor cell lysis by CTLs is an efferent stage of anti-tumor activity. Two types of T cells recognize different molecular structures, helper T cells bind to a class II molecule with an antigen but CTLs attach to a class I molecule plus an antigen [Fig. (1)]. What kind of antigenic structures is recognized by T cells ? Crystal structures of class I and class II molecules of MHC revealed that a short peptide derived from an antigenic molecule is embedded in a groove of MHC molecules [12,13]. The complex configuration is in fact the target structure recognized by T cells [14]. What kind of molecules can be a tumor antigen or a tumor-associated antigen ? Several types of peptides are clarified in the case of melanoma. Firstly, differentiation antigens that are preferentially expressed in tumor cells but are also found in normal tissues at a certain differentiation stage. This category includes MART-1 and gp100 [15]. Secondly, proteins that are expressed even in normal cells but have a mutation(s) in tumor cells. Thirdly, novel proteins that are generated by alternative transcription or are completely new gene products. At present, several melanoma-associated antigens such as gp100 and tyrosinase are known to be the targets of melanoma-specific CTLs [16], but a few peptides are known as tumor antigens in other human tumors [17].

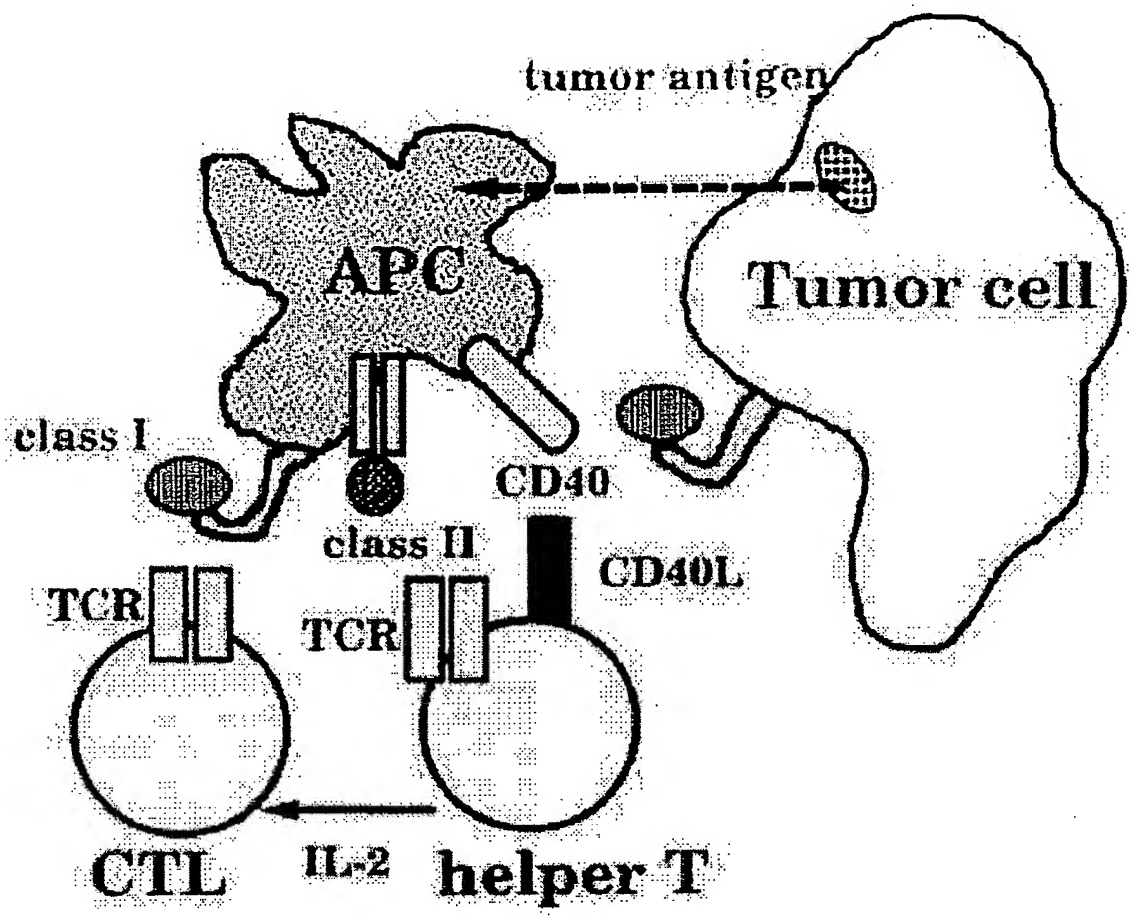

Fig. (1). Interaction between APC and CTLs or helper T cells. Tumor antigen(s) from tumors can be incorporated into APC and a short peptide is presented on cell surface with class II molecules. Helper T cells recognize the complex through TCR and secrete cytokines such as IL-2. APC also present a short peptide that is embedded in class I molecules. CTLs stimulated by the complex undergo proliferation. A signal pathway through CD40/CD40L interaction is important for APC activation.

The way of antigen processing is tightly regulated. Peptides derived from endogenous cytosol proteins are generated by proteasome and are exclusively associated with class I molecules within the lumen of endoplasmic reticulum. They are then transported to cell surface [18]. Instead, exogenous proteins are incorporated into cytoplasm via endosome where limited proteolysis occurs, and antigen peptides are assembled with class II molecules in the late endosome [19]. These mechanisms raise an important problem, because APC have a dual action to present a tumor antigen to helper T cells and to activate CTLs that are specific to the tumor antigen [Fig. (1)]. Since tumor antigen(s) are exogenous to APC, the presentation of the peptide derived from tumor cells should be mediated by the class II pathway. However, APC have to carry the peptides through the class I pathway in order to activate CTLs. Recently, this problem has been solved through the analysis of dendritic cells (DC). DC are professional at antigen presentation and may be the only APC that can stimulate resting or naive T cells, and can consequently initiate CTL-mediated immune responses *in vivo* [20]. Therefore, antigen processing within DC is crucial for anti-tumor responses. Besides antigen presentation by the class II pathway, DC have developed a unique membrane traffic system, endosome-to-cytosol transport [21]. Accordingly, internalized antigens can gain access to the conventional class I pathway. This novel traffic system can confer DC on a specific role in CTL-mediated target cell killing, although DC are heterogeneous in their origin. They are composed of at least two subpopulations, one in the myeloid lineage including Langerhans cells and the other in the lymphoid lineage [22,23].

Th1 AND Th2 EFFECTOR FUNCTION

Combinatory actions of cytokines can contribute to each cellular and humoral step

toward tumor cell killing, but the other sets of cytokines may counteract the actions, which may induce immune tolerance to tumors. Since a single or a few cytokines can be practically utilized in cytokine therapy, to know how cytokines affect immune responses is important. To activate and endow APC (DC) competent for antigen presentation, stimulation of CD40 molecules on their surface with CD40 ligand (CD40L) that are mainly expressed on T cells is a crucial point [Fig. (1)] [24]. Interaction between costimulatory molecules, CD80 (B7-1) or CD86 (B7-2) expressed on DC, and CD28 on T cells, results in proliferation of T cells and production of inflammatory cytokines such as tumor necrosis factor (TNF)-α [25], which is involved in the upregulated expression of class I and class II molecules. The interaction also increases the secretion of interleukin (IL)-2, interferon (IFN)-α and granulocyte macrophage-colony stimulating factor (GM-CSF). In addition, IL-12 that is also released from DC, contributes to the maturation of T cells.

In the process of expansion of CTLs, they need a help from $CD4^+$ T cells , IL-2 and/or IFN-γ. Maturation of helper T cells is necessary to be competent to produce such cytokines and the function of $CD4^+$ helper T cells is indispensable for the development of CTLs. Based on the pattern of secreted cytokines, $CD4^+$ helper T cells are classified into two distinct subpopulations, type 1 helper T (Th1) and type 2 helper T (Th2) cells [26]. Th1 cells produce IL-2, IFN-γ and TNF-β, whereas Th2 cells secrete IL-3, IL-4, IL-5, IL-10 and TGF-β in general. However, a sensitive assay for cytokine production in individual cells has come to show that many $CD4^+$ T cells cannot easily be grouped into Th1 or Th2 subsets based on the original criteria. At a single cell level, $CD4^+$ T cells exhibit a heterogeneous pattern in secreting various combinations of IL-2, IL-4, IL-10, IFN- γ and TGF-β. Although the T cell subsets may not be categorized by their profile of cytokine production, many experimental studies or naturally occurring immune responses support that the pattern of cytokine production can be linked with Th1 or Th2 dominant immune state. Th1-type immune responses are often associated with inflammation and defense reactions including cytolytic activity, whereas Th2-type responses are antibody-mediated immunity. Correlation of the function of each cytokine with the classified immune state, Th1- or Th2-type response, is not significantly strong, but in general immunological effect of $CD4^+$ T cells can be functionally divided into Th1- or Th2-type. Since Th1-type cytokines promote the maturation and proliferation of CTLs, these cytokines are candidate molecules for therapy in order to enhance cell-mediated immunity. In contrast, Th2-type cytokines rather inhibit inflammatory reactions. Interaction between the two types of responses is reciprocal to each other; Th1-type cytokines are inhibitory to Th2-type responses and vice versa. In that sense, to suppress the secretion of Th2-type cytokines is favorable to the promotion of anti-tumor activity. If the whole immune response is possibly evaluated by relative ratio between Th1-type and Th2-type activity, to shift the immune balance to Th1 dominant state will contribute to the enhancement of anti-tumor responses.

Generation of Th1-type T cells is thereby crucial for cell-mediated immunity. Naive $CD4^+$ T cells can be differentiated into Th1 or Th2 cells, depending on the antigen presented by APC or cytokines concentrated in microenvironment. IL-4 plays a key role in the early phase of this differentiation process and it favors the development of Th2 in the latter phase of the differentiation. In contrast, IL-12 [27] or IL-18 (previously described as IFN-γ inducing factor) [28] can synergistically support the development of Th1 cells. Although IL-12 or IL-18 does not belong to Th1-type cytokines, their biological functions are closely related with Th1-type immunity. They induce the secretion of IFN-γ and augment the proliferation of Th1 and NK cells. These cytokines seem to work synergistically in many facets of Th1 immunity, although the distribution of each receptor is different. Therefore, these Th1-inducing cytokines are also candidate molecules to be used for cancer treatment.

CYTOKINE THERAPY

What kind of cytokine is effective to induce host immune defense system ? The choice of cytokine(s) for cancer therapy is not easy, because the effectiveness can be dependent on many factors such as the extent of immune suppression in cancer patient and antigenicity of tumor cells. However, it is reasonable to speculate that Th1-type cytokines such as IL-2 and IFN-γ would be favorable to anti-tumor responses, promoting systemic cell-mediated responses. On the other hand, GM-CSF and G-CSF, although they are not directly involved in cytotoxic effect, can circumvent suppressed hematopoiesis that is caused by high-dose chemotherapy and have been evaluated as being clinically beneficial [29]. This

is another category of cytokine-mediated cancer therapy.

Cytokine therapy for cancer treatment was initially tested by the use of recombinant proteins, but the toxic activities accompanied by *in vivo* administration of the proteins became soon apparent and serious adverse actions hampered extensive clinical application of the recombinant proteins. However, subsequent studies have reported that expression of cytokine gene(s) in tumor cells could elicit potent anti-tumor responses. It may be unimportant to discriminate between gene-based therapy and protein-based therapy, because combinatory treatment using both cytokine genes and recombinant proteins, and transduced cell-based therapy are often tried. The advantage of recombinant cytokines is the homogeneity of products and consequently we can measure the biological effects by using standardized amounts of the protein. Recombinant proteins are stable and pure in their quality. However, they are relatively expensive and the half life *in vivo* is unfortunately short in general. In contrast, gene therapy, expression of cytokine gene in tumor cells, is relatively less costly and the advance in vector technology has enabled us to examine the therapeutic effect of *in vivo* gene transfer. However, it is difficult to control the amounts of secreted cytokines and adverse reactions induced by the vector system cannot be avoidable. Nevertheless, the gene therapy using cytokine genes is in fact the most frequently approved trials among gene therapy for cancer. In this review, I firstly describe the cancer therapy using recombinant proteins and then the recent progress in cytokine gene therapy.

IL-2-BASED IMMUNOTHERAPY

Among cytokines, IL-2 has been widely tested for its anti-tumor activity. However, systemic application of IL-2 induces various adverse effects such as vascular leakage syndrome and marked fluid retention in extravascular space. Hence it cannot be an appropriate procedure, to use high-dose IL-2. A short half-life of IL-2 in human body (less than 10 min) is also a limited factor. Therefore, IL-2 is mainly used ex vivo to expand lymphokine-activated killer (LAK) cells in which T and NK cells are included. Most of the studies using recombinant IL-2 and/or LAK cells showed that the anti-tumor effects by IL-2 or LAK cells alone were not potent enough to suppress the growth of tumors except highly immunogenic tumors.

Background and Experimental Studies

IL-2 has been originally identified as a mitogenic factor of T cells. Identification of the gene encoding IL-2 molecule showed that it is 15.5 kDa glycoprotein. The receptor of IL-2 was identified to have three subunit molecules, α-, β- and γ-chains. The molecular organization of the receptor, consisting of heterodimeric structure, is strictly related with the binding affinity to IL-2. The biological function of IL-2 is basically to transduce a growth signal to T and NK cells [30]. Although only 10 % of NK cells express high-affinity IL-2 receptors, the remaining 90% can also bind IL-2 [31]. In response to exogenous IL-2, NK cells secrete several cytokines such as IFN-γ, GM-CSF and TNF-α. Resting T cells do not express IL-2 receptors and do not respond to IL-2, unless they receive TCR-mediated signalings. Once T cells are activated by antigens or mitogenic signals, they express IL-2 receptors efficiently and consequently IL-2 enhances the proliferation of activated T cells. This mechanism is advantageous to selective expansion of antigen-primed T cells and eventually contributes to the establishment of immunological T cell-memory.

A variety of therapeutic approaches to treat tumor-bearing mice using recombinant IL-2 has been examined and the studies showed anti-tumor activities by IL-2 to a certain extent [32,33]. To obtain better therapeutic effects, maintenance of *in vivo* IL-2 concentration at a certain level is important, because IL-2 is rapidly cleared from body [34]. While continuous infusion of IL-2 is a choice of therapy, another approach is to activate and expand immunocompetent cells by IL-2 *in vitro* and to transfer them into tumor-bearing mice. When peripheral blood or spleen cells are cultured in the presence of high concentrations of IL-2, a heterogeneous population of T and NK cells are activated to proliferate extensively. These cells, named LAK cells, can destroy a number of tumor cells nonspecifically. The lytic activity of LAK cells are mostly attributable to the activated NK-lineage cells [35,36]. The spectrum of targets by LAK cells is not only tumor cells but virus-infected cells, hapten-modified cells, allogenic cells and even normal lymphocytes [37,38]. The destruction of endothelial cells can be recognized in part as an adverse effect by LAK cell therapy. Preclinical studies have been conducted to test the LAK cell-mediated anti-tumor effect in tumor-bearing animals [39,40]. Administration of LAK cells generated from spleen cells into mice that had tumors and/or metastatic foci could prolong the survival of mice and suppress the expansion of

metastasis. The adoptive immunotherapy has been effective in most of the experimental cases tested and combination of recombinant IL-2 administration and LAK cells infusion produced better effect than either treatment alone [40].

Clinical Application

Initial application of IL-2 to cancer therapy was explored by systemic administration of natural or recombinant IL-2 into patients of immunogenic tumors such as melanoma and renal cell carcinoma. The observed clinical benefits were not significant, partly because the patients who received IL-2-mediated therapy were in advance stages and to whom standard therapy was ineffective [41]. Combination of systemic administration of IL-2 and LAK cells gave rise to better clinical effects than the therapy with IL-2 alone in melanoma and renal cell carcinoma patients [42]. The overall response rate by IL-2 coupled with LAK cells infusion was reported to be 10-20 % including partial response [42-44]. Thereafter, LAK cells infusion together with chemotherapy and/or administration of cytokines has been tried in various cancer cases. However, clinical outcomes by the strategy turned out to be not impressive [45-47], although better therapeutic effect could be observed to some extent [48]. Nevertheless, migration of lymphocytic cells into primary and metastatic foci after the treatment was noticed and these data suggested that LAK cells could infiltrate into the tumors and work for tumor destruction. To improve the therapeutic effect, tumor-infiltrating lymphocytes (TILs) [49,50] were used. TILs are cells that infiltrate into growing tumors and are obtained from tumor cell suspension. They can proliferate *in vitro* with IL-2 and become predominant in culture after several weeks [50]. Since TILs are considered to be able to migrate into tumor tissues, they may recognize putative tumor antigen(s) or tumor-associated antigen(s). In fact, preclinical studies revealed that TILs could show better therapeutic effect than LAK cells [49]. Most of TILs studied were composed of $CD3^+$ T cells, in contrast to LAK cells. *In vitro* studies also demonstrated that cytotoxic activity of TILs was MHC-restricted, suggesting the presence of tumor antigen(s) expressed on autologous tumors [51]. Migration of infused TILs, after expanded ex vivo, into the targeted tumors was in fact experimentally shown by marking TILs with a radioisotope [52] or the neomycin phosphotransferase gene [53]. Repopulation of TILs to tumors and their lytic action demonstrated the feasibility of TIL-mediated cell therapy. These evidences also indicate a potential use of gene-modified TILs. The majority of the cases who received TIL-mediated immunotherapy in early studies were melanoma and renal cell carcinoma patients, who did not respond to any conventional therapy. Objective regression of tumor foci was observed and the clinical outcome seemed to be promising [54]. However, latter studies showed that significant responses to the therapy were not produced in most cases [55,56].

During the clinical trials, a number of drawbacks associated with IL-2-mediated therapy soon became apparent. The toxicity was in general derived from inflammatory reactions. These toxic reactions were dose-dependent of IL-2 used [57] and belonged to a delayed-type hypersensitivity response. The adverse reactions include fever, malaise, myalgia, nausea and hypotension. These unfavorable effects are not totally due to IL-2 but due to secondary cytokines secreted from activated cells by IL-2, because the onset of toxicity effect was delayed for several hours. NK cells, for example, may produce a number of cytokines such as IFN-γ and GM-CSF, after they are activated. Sudden release of these cytokines consequently activates other cell populations such as monocytes and these cells release proinflammatory cytokines including IL-1, IL-6 and TNF-α. Systemic administration of IL-2 also increase the permeability of blood vessels called vascular leakage syndrome [58]. The phenomenon is not due to hemodynamic changes but in part due to platelet and neutrophil's adhesion to endothelium [59]. Infusion of LAK cells also have similar side effects such as fever and malaise, but most of the patients can tolerate these adverse reactions.

ANTI-TUMOR EFFECT OF IFN AND TNF-α

Mechanism of Anti-tumor Effect

Three types of IFN, IFN-α, -β and -γ, have been identified and they have pleiotropic biological activities. The mechanism of IFN-mediated anti-tumor effects is modulation of cell-mediated immunity. In particular, IFN-γ can augment cytotoxic function of CTLs, NK cells and activate monocytes/macrophages [60,61]. Antibody-mediated cytotoxicity is also enhanced. Besides the activation of immunocompetent cells, IFN-γ can augment the expression of class I and II molecules of MHC. The elevated expression of class I antigens on tumor cells increases the antigenicity of tumors, facilitating the recognition

of tumor cells by CTLs [62]. Class II antigens are expressed on APC that can incorporate tumor antigen(s) and their enhanced expression promotes the processing of tumor antigen(s) [Fig. (1)]. Another biological character of IFN-γ is to activate the transcription of other genes involved in an immune response and signal transduction. Among the genes activated, IFN-inducible protein 10 (IP-10) and monokine induced by interferon-γ (Mig) can contribute to anti-tumor activity [63,64]. These chemokines suppress neovascularization of tumor masses and in fact part of anti-tumor effect of IL-12 is mediated by IP-10 and Mig induced [64].

Clinical Study

Administration of IFN-α caused a good anti-tumor effect to hematological malignancies such as hairy-cell leukemia [65,66], lymphomas [67,68] and chronic myelogenous leukemia [69,70]. IFN-α can inhibit cell growth by inducing G1-arrest but the mechanism of IFN-α-mediated anti-tumor effect remains controversial [71,72]. It has been also used for melanoma and metastatic renal cell carcinoma in combination with other cytokines such as IL-2 [73]. Clinical responses to IFN-α were not clearly observed [74] but the combination therapies using anti-cancer agents and/or IL-2 could improve the clinical outcome. As an adjuvant therapy, IFN-α is being used for hematological malignancy [75-77]. In melanoma cases, high-dose IFN-α was examined for its feasibility in clinical use. Compared with a low-dose of IFN-α, the patients who received high-doses of IFN-α have shown good clinical responses. However, the toxicity generated by high-doses of IFN-α was so strong that only one half of the cases could continue the therapy [78]. The toxicity includes asthenia, neutropenia, and nausea/vomiting [79]. In renal cell carcinoma, remarkable anti-tumor effects were not observed [80,81] but in some cases IFN-α administration can contribute to prolonged survival of the patients [82]. IFN-γ has been also used for advanced cancer patients in combination with IL-2 and TNF-α [83-85]. Although immunological parameters of the patients improved, significant clinical benefits were not seen [84,86].

TNF-α is an inflammatory cytokine and is secreted from many cell types including macrophages and polymorphonuclear cells [87]. It has anti-tumor activity through direct cytotoxic action which is primarily due to apoptosis. TNF-α also promotes intravascular thrombosis within tumor tissues, which leads to necrosis of tumors, and activates immunocompetent cells including neutrophils, macrophages and NK cells [87]. Activation of these cells in turn induce the production of inflammatory cytokine such as IL-1, IL-6 and IL-8 and upregulation of adhesion molecules on cell surface. These secondary reactions can further recruit immunocompetent cells around tumors and consequently enhance tumor destruction.

Administration of TNF-α into tumor-bearing animals prolonged their survival [88]. Clinical trials by combinatory administration of TNF-α and other cytokines were tested [89]. Septic shock and thrombocytopenia were major limiting factors for its clinical application and no apparent anti-tumor effects were observed after systemic administration [90,91], although local application of TNF-α into metastatic foci may be beneficial [92].

CYTOKINE GENE THERAPY USING Th1-TYPE CYTOKINES

While administration of recombinant cytokine(s) can produce to some extent anti-tumor effects or immunological changes in patients, the toxic reactions preclude the use of large amounts of the recombinant proteins. The other idea is to transfer cytokine gene(s) in tumors and to secrete the cytokine into the vicinity of tumors. The objective of this gene-based therapy is the same as that of protein-based therapy, to activate host immune system with a few kinds of cytokines. Induction of tumor-specific CTLs and generation of potent cytotoxicity are an ultimate purpose of immune therapy. For that purpose, identification of tumor antigen(s) is ideal and tumor-specific CTLs can be induced by immunization with peptide(s) derived from tumor antigens. However, it is often difficult to identify tumor antigens in human cancer. Instead, cytokine gene therapy does not have to reveal such antigen(s). Although immunization with tumor antigens and use of cytokine to enhance immune response against the antigens is preferable, generation of cytokine-mediated immune response is at present the subject to be explored. An advantage of cytokine gene-based therapy is to avoid the toxic reactions induced by systemic administration of cytokine proteins. Transgene-derived cytokines can continuously stimulate an immune system in contrast to recombinant proteins that are easily degraded *in vivo*. However, to measure the local concentration of cytokines in the microenviron-

ment around tumor tissues is often difficult. Moreover, *in vivo* transfer of cytokine gene needs novel vector systems.

The reason why cytokine-secreting tumors can induce systemic anti-tumor immunity is not fully understood. It depends on the cytokines used and experimental models. One of the reasons is that tumor cells may present tumor antigen(s) by themselves and facilitate the activation of helper T cells, when tumor cells happen to express class II molecules. Cytokine secreted from tumors may change the expression of adhesion molecules on endothelium or stroma neighboring tumors and consequently it promotes the recruitment of T cells into the tumor masses. T cells that migrate into tumors can proliferate owing to the cytokine in local environment. Destruction of tumors accompanied by local inflammatory reactions can be facilitated by cytokines and a cascade of a local immune response that leads to systemic immune responses will be generated. Another reason is related to inhibition of neoangiogenesis in tumors. Although the precise mechanism remains ambiguous, a number of experimental models have shown that local secretion of cytokine(s) from tumor cells can induce effective anti-tumor responses [93].

Implantation of cytokine gene-modified tumor cells into syngeneic mice is an initial step to examine the effectiveness of cytokine gene therapy. Using a drug resistance marker and an enzyme-linked immunosorbent assay, cytokine-producing tumor cells can be easily established after gene transfer. *In vitro* proliferation capacity of cytokine-producing tumor cells and the expression levels of MHC class I and class II molecules should be the same as those of parental cells. In order to know which cytokines can induce anti-tumor effects, various cytokine genes and combination of the genes have been examined in animal experiments. One notion is to express Th1-type cytokine genes, which results in enhancing cell-mediated immune responses. We and other showed that Th1-type cytokines, when secreted from tumor cells, can elicit systemic immune responses against cytokine-producing tumors [94-98]. IL-2- or IFN-γ-producing cells were inoculated into syngeneic immunocompetent mice. The representative data using IL-2-producing tumor cells showed that the cytokine-producing tumors, depending on the amounts of secreted cytokine, regressed spontaneously after they had developed small tumors [98,99]. The mice that had rejected cytokine-producing tumor cells developed tumor-specific protective immunity; the mice could rejected untransduced parental tumors that were subsequently inoculated, even when they were administered with tumorigenic cell number; however, they accepted irrelevant syngeneic tumors and the growth of the irrelevant tumors remained the same as that of the irrelevant tumors inoculated in naive mice [98,100]. The observed anti-tumor effect suggests the generation of tumor-specific CTLs in the mice that were inoculated with IL-2-producer cells. In this regard, the induction of CTLs has been shown *in vitro* and *in vivo* [96].

In contrast to Th1-type cytokines, when Th2-type cytokines were engineered to secrete from tumor cells, the rejection of inoculated tumor cells was not observed. In our and other experiments, murine tumors that secrete IL-4, IL-6 or IL-10 developed tumors in syngeneic mice and anti-tumor effects were not evoked [101,102]. Moreover, in the case of IL-10-producing tumors, the tumor size inoculated in syngeneic immunocompetent mice was significantly larger compared with parental tumor size (unpublished data). Since IL-10 is a cytokine that suppresses cell-mediated immune responses [103], local secretion of IL-10 may trigger escape mechanism of tumors from an immune system. In fact, the concentration of IL-10 can be an unfavorable prognostic factor in cancer patients [104,105]. Although we observed that tumor cells secreting Th2-type cytokines were not rejected, other studies reported that Th2-type cytokine-producing tumor cells elicited potent anti-tumor responses, when inoculated in mice [106-109]. The anti-tumor response induced by Th2-type cytokines is attributable partly to the inhibitory action of neoangiogenesis [108] or to nonspecific inflammatory reactions [106]. IL-10 was also reported to inhibit the function of macrophages that may suppress cell-mediated immune responses [110]. Regulation of other molecules such as TGF-β and inducible nitric oxide synthase generated by Th2-type cytokines can contribute to the anti-tumor responses [107,109]. In the case of IL-4-producing tumors, suppressed neovascularization by IL-4 is regarded to be a major reason for anti-tumor activity by the local secretion of IL-4 [111,112]. In spite of these contradictory reports that expression of Th2-type cytokines in tumor cells can induce loss of tumorigenicity, Th2-type cytokine-producers do not in general show anti-tumor effects *in vivo*.

To confirm whether expression of Th1-type cytokines in tumor cells can generate effective anti-tumor responses, we and others tested other

cytokines that are functionally Th1-type-equivalent. IL-15, a novel cytokine which shares some of the IL-2 receptor components (β and γ chain) [113], have similar biological functions as IL-2 has such as support for T cell growth [114,115]. Recently, a human IL-15-specific α subunit gene has been cloned and this subunit is responsible for high-affinity binding of IL-15 [116]. The tissue distribution of IL-15 receptors is wider than that of IL-2 receptors: not only on hematopoietic cells but on endothelial cells [116]. Several toxic reactions such as vascular leakage syndrome that were observed in preclinical and clinical studies using high-dose IL-2 may be due to the activation of IL-15-mediated signaling pathways. The broad distribution of the α subunit can be related with differential biological significance between IL-2 and IL-15. One of the characteristics of IL-15 is that it is a prerequisite factor for development and maturation of NK cells [117,118]. The precise gene regulation of IL-15 is complicated and the amounts of secreted IL-15 also depend on its splicing pattern [119,120].

Anti-tumor effects of IL-15 was demonstrated by expressing IL-15 gene in tumor cells. Inoculation of IL-15-producing tumor cells in syngeneic animals were rejected in syngeneic immunocompetent mice [121,122]. In immunosuppressed nude and severe combined immunodeficient (SCID) mice, the inoculated tumors were not rejected but showed growth retardation, suggesting that the anti-tumor activity was diminished in mature T cell-defective, B cell-defective and NK1.1$^+$T cell-defective conditions [123]. These results showed that not only NK cells but other cells including T cells are important to produce anti-tumor effects. The mice that had rejected IL-15-producing tumors were also resistant to subsequent challenge of lethal dose of parental cells, and this protective immunity was antigen-specific.

It is important to know the cell types that are involved in anti-tumor responses. Inoculation of cytokine-producing tumors into the mice whose specific subpopulation(s) is deleted by administering antibody against the cell type, immunocompromised mice or specific gene-deficient mice by homologous recombination can tell us the cell population(s). What cell type is necessary to generate an anti-tumor response differs among experimental models and cytokines used. In most of IL-2-producing tumors, anti-tumor effects are attributable not only to CD8$^+$ T and CD4$^+$ T cells but to NK cells, granulocytes or macrophages [96,124,125]. These non-T cells are known to express IL-2 receptors. Many reports showed that transduction of IL-2 gene in tumors could effectively induce anti-tumor effects even in nude and SCID mice [124, 126]. Interestingly, tumor-specific protective immunity is induced in nude mice which are deficient in αβ T cells [126]. The protective immunity induced is probably due to γδ T or NK1.1$^+$T cells which are intact in nude mice.

A histological examination of cytokine-producing tumors does not always show the cell types which are responsible for anti-tumor effects. Acquisition of systemic immunity and *in vitro* analysis of immunocompetent mice demonstrated that mature T cells, including αβ and γδ T cells, are involved in anti-tumor activities. However, immunohistochemical staining of cytokine-producing tumors did not clearly reveal the infiltration of T cells into tumor masses. Migration of macrophages is often evident. Macrophage accumulations around the tumors may reflect their scavenger function to digest necrotic tumors. The accumulations can also contribute to antigen presentation of putative tumor antigen(s). Histological findings do not often agree with sequential immunological processes, but tumor necrosis and infiltration of immunocompetent cells are frequently observed in tumors in their regressing phase.

NOVEL CYTOKINES THAT CAN MEDIATE IFN-γ PRODUCTION

IL-12 and IL-18 belong to a new category of Th1-type-equivalent cytokines [27,28]. These cytokines can induce the production of IFN-γ and promote the differentiation of Th0-type, undifferentiated, immature T cells into Th1-type cells. Both cytokines serve similar biological functions in several aspects, but possess distinctive properties, some of which are derived from the difference of their receptor distribution in tissues. Therapeutic effects by recombinant IL-12 or IL-18 were tested in a number of experimental animal models [127-129]. These results showed that recombinant IL-12 can eliminate established tumors but this dramatic effect cannot be always produced. It depends on the type of tumors. What kind of factors is involved in influencing the effectiveness of IL-12 is not clear. However, several reports showed that migration of T cell into the tumor masses is critical. Secretion of IL-12 often upregulates the expression of adhesion molecules on vascular endothelium such as intracellular adhesion molecule-1 or vascular cell

adhesion molecule [130]. This increased expression of adhesion molecules in a vascular system within tumors and peritumoral area can facilitate the migration of immunocompetent cells. When the infiltration of immunocompetent cells is efficiently induced by IL-12, established tumors are eliminated [130]. However, if the upregulation is not induced, IL-12 fails to show significant anti-tumor effects. Administration of recombinant IL-12 into advanced cancer patients has been examined for its clinical value [131,132], but significant liver damages hampered further clinical trials and gene transfer of IL-12 gene is now being tested.

IL-18 was initially described as a molecule that can induce IFN-γ secretion. The secretion of IL-18 from cells requires a specific enzyme, caspase-1, as found in the case of IL-1β secretion [133,134]. The function of IL-18 is diverse. For example, it enhances NK activity and the expression of IL-2 receptors on T cells, which are similar to the biological activities that IL-12 has. The distinctive functional differences between IL-18 and IL-12 are not clearly understood and most of the experimental studies showed that IL-18 can synergize with IL-12 [135]. However, distribution of respective receptors is different and IL-12 induces and augments the expression of the IL-18 receptor [136]. Since both cytokines induce IFN-γ, several studies reported that the molecules induced by IFN-γ can be a key modulator to determine the effectiveness of the anti-tumor activity by IL-12 or IL-18. The candidate molecules are chemokines such as IP-10 and Mig [64]. These molecules suppress the angiogenesis within tumor masses and work as a chemotactic factor to recruit inflammatory cells around tumors [137]. These chemokines can also enhance antigen-presentation activity of APC [138].

Recently, $NK1.1^+T$ cells were reported to be an indispensable cell population in IL-12-mediated anti-tumor activity. Cui *et al.* showed that $NK1.1^+T$-deficient mice cannot abrogate inoculated tumors with IL-12 but the Vα14-gene transgenic mice that lack the recombination activating gene (thereby have only $NK1.1^+T$ cells) can restore the IL-12-mediated anti-tumor activity [139]. These data does not rule out the involvement of T and NK cells in IL-12-mediated anti-tumor responses, but clearly proves that $NK1.1^+T$ is essential target cells of IL-12.

Expression of IL-12 or IL-18 gene in tumor cells was effective to induce systemic anti-tumor activities [140,141] and these studies also supported the idea that Th1-type cytokine genes are useful in cancer gene therapy. Since IL-12 is heterodimeric (inducible p35 and constitutively expressing p40 subunit), recombinant protein, instead of coexpression of the two genes encoding the subunits, was initially tested. Preclinical experiments demonstrated strong anti-tumor activities. However, IL-12 is relatively toxic and induces severe liver damages as described [131,132]. In gene therapy, both p35 and p40 gene are linked with internal ribosomal entry site and consequently coexpression of both subunits within cells becomes possible [142]. The target cells in which IL-12 may be expressed are not restricted to tumor cells. Recent data showed that IL-12-secreting DC, when injected in the vicinity of tumor cells, are quite effective for tumor eradication [143], because IL-12 secreted from DC plays an important role in antigen presentation particularly through CD40/CD40L pathway [144].

TUMOR VACCINE USING CYTOKINE-PRODUCING CELLS

Since *in vivo* transduction of cytokine genes into tumor cells has potential problems associated with the use of vectors, tumor cells that were surgically resected and transduced ex vivo were used as a tumor vaccine. Irradiated cytokine-producing tumor cells were injected into naive animals to immunize them and they were challenged with parental tumor cells [145]. A certain dose of irradiation that could eliminate the proliferating capacity of tumor cells but relatively maintained the cytokine production was selected. Irrespective of cell-types, 10-60 Gy-irradiation in general depletes the growth capability but does not seriously damage the cytokine productivity in murine tumors [146]. In some cases, irradiation transiently increased cytokine secretion due to the leakage of cytokines through damaged cell membrane [147]. Immunization with irradiated cytokine-producing tumor cells can generate systemic protective immunity. This vaccine effect is dependent on the immunogenicity of parental tumors. Irradiated parental tumors, when injected into immunocompetent animals, can sometimes generate systemic immunity, and cytokine secretion from the tumors efficiently increases the vaccine effect. This immunization procedure is effective even to established tumors and can decrease the number of preexisting metastatic foci [145, 148]. Expression of GM-CSF gene in tumor cells do not generally induce systemic immunity, when the tumor cells are inoculated. However, when irradiated GM-CSF-transduced tumor cells

are injected, they can elicit potent immunity [149]. The reason why GM-CSF-producing tumors are effective as a tumor vaccine is not clear, but it is probably due to the increased antigen presentation by secreted GM-CSF.

TARGETTING TUMORS WITH CYTOKINE GENE THERAPY

Efficacy of cytokine gene therapy has been examined in a number of tumor models, including the tumors that are not immunogenic. In the brain, immune responses are not evoked sufficiently since brain is considered to be an immunologically privileged site. One of the reasons is that immunocytes are not able to access brain well because of the blood-brain barrier. Immune responses against intracranial tumors are not as powerful as those against subcutaneous tumors. Furthermore, production of IL-2 from brain tumors can induce brain edema, resulting inefficient anti-tumor effects with cytokine gene therapy [150]. However, an immune response generated by subcutaneous immunization of IL-2-secreting brain tumor cells can induce adequate immunity to the same brain tumors that develops subsequently [151]. The histological examination of the intracranial tumor revealed that microglial responses, which reflect inflammation, and infiltration of macrophages and T cells were observed [152, unpublished data]. These data contradict the previous findings but show that the blood-brain barrier can allow the migration of immunocompetent cells into the brain, when brain tumor develops.

The anti-tumor effects are affected by the administration route of cytokine gene-modified tumor cells. Subcutaneous inoculation of the modified tumors can effectively induce systemic immunity, but administration of the tumor cells into intraperitoneal cavity is less effective. Intravenous injection of the tumors scarcely shows anti-tumor effects. Potency of induced immunity is partly attributable to the degree of antigen presentation. APC such as Langerhans cells are abundant in subcutaneous tissues compared with peritoneal cavity or lung and they may process the tumor antigen more efficiently than the APC in other tissues. Accessibility of APC to the transduced tumors is a key to the effectiveness of cytokine gene therapy.

Is it possible to apply the results of animal studies to human cases? Most of the tested animals were small in size compared with humans, and the most important issue is that human tumors develop spontaneously but animal tumors are experimentally transplanted. Long-term culture *in vitro* often causes the mutations of a number of genes and phenotypic changes in tumor cells. These alterations affect the properties of the tumor cell lines. Modification of the expression levels of major and minor histocompatibility complexes undoubtedly affects the immunogenicity of the tumors. In that sense, all of the cell lines used in preclinical studies are immunogenic in contrast to non-immunogenic tumors developed in patients. Since humans are not inbred animals, genetic variations among patients influence the tumor-host interactions. Accordingly, the difference in class II gene expression among patients and the binding affinity of antigen peptides to the class II molecules affects the efficiency of antigen processing. In other words, a differential immune response of individual patients greatly modifies the clinical outcome, even when the tumors originate in the same tissue and have the same histopathological features.

CLINICAL APPLICATIONS OF CYTOKINE GENE THERAPY

Vectors for Gene Transfer

A number of preclinical studies revealed that a certain cytokine gene, when expressed in tumor cells, can produce anti-tumor effects. The efficacy is dependent on many factors such as the antigenicity of tumors and the amounts of cytokine produced. To apply cytokine gene therapy to the treatment of cancer, we have to consider several factors which are critical for successful trials: what kind of cytokine(s) is effective for a particular type of cancer, optimal amounts of secreted cytokine, and how long the production of cytokine should continue. It is also important to evaluate the immunological conditions of patients. Since specific interaction between the patient's immune system and the tumor is difficult to analyze in animal studies, randomized clinical trials are the way to know the efficacy of cytokine gene therapy [153].

There are several technical issues to be settled for the clinical application of cytokine gene therapy; transduction efficiency and safety of vectors. Several methods of gene transfer have been tested. For the standpoint of safety, DNA-conjugated liposome is the best method. Encapsulation of DNA with cationic liposome greatly enhances its incorporation into tumor cells

but further improvement on the transduction efficiency is needed [154]. Retroviral vectors that possess several mutations in the genes in order to avoid unnecessary production of wild-type virus have been frequently used as a vehicle in a number of clinical trials [155]. Since retrovirus infects the cells which are proliferating, preferential integration of the therapeutic gene into tumor cells is an advantage to circumvent the gene expression in non-tumorous tissues. One of the major concerns about retroviral vectors is their potentiality to induce tumorigenesis. Random integration of retrovirus into the chromosome of recipient cells may generate unwanted activation of oncogenes or inactivation of tumor suppressor genes, depending on the integration sites. Although a classical retroviral vector was reported to induce leukemogenesis in the tested primates, there is no incidence reported that the administered retroviral vectors induced tumorigenesis in the patients who received retrovirus-mediated gene therapy.

The major obstacle that retroviral vectors possess is their insufficient transduction rate. Preparation of high titer virus is also technically difficult at present. Moreover, retrovirus is not stable particularly *in vivo*, because retrovirus is susceptible to human complement. Recently, packaging cell lines form human cells are now under development to increase the transduction rate, since the retrovirus prepared from the packaging cells of human origin is resistant to complement-mediated inactivation of retrovirus. Nowadays the usage frequency of retroviral vectors as a clinical-grade vector for cancer gene therapy is becoming lower. Use of retrovirus-producing packaging cells is an alternative choice of vehicle for gene transfer. However, packaging cells derived from murine origin are allogenic and rapidly killed by human immune system. The transduction efficiency is also quite low.

Adenoviral vector has an advantage in high transduction efficiency *in vitro* [156]. However, an immune response to adenovirus, when administered *in vivo*, is induced. The use of adenoviral vectors in human is further complicated by the fact that most of the individuals have immunity to adenovirus. Antibody against adenovirus is further developed in the patients who received adenovirus and CTLs for epitopes present on the viral structural proteins are also generated [157]. It is controversial as to whether these neutralizing antibodies and CTLs can deteriorate the transduction efficiency, because sufficient transduction by adenovirus can be achieved in spite of the presence of the antibody and the CTLs [158]. For most of the cancer gene therapies, transient expression of therapeutic genes is enough to produce potent biological consequences. For that reason, adenovirus is a frequently used vector for cancer gene therapy at present. However, administration of high-dose adenovirus can induce toxic responses in the recipients. In particular, adenovirus preferentially accumulates into the liver. Although systemic administration of adenovirus often induces liver damages [159], high transduction efficiency of adenovirus in the liver may be favorable for treatment of hepatoma. Adverse reactions in the case of local administration of adenovirus is often mild and well tolerable to most of patients.

Clinical Trials

More than 2200 cancer patients in the world had received gene therapy until September, 1999 and this number is about 70% of total patients who received gene therapy. Scope of targeted tumors is widely distributed and various combinations of therapeutic genes are now being investigated. Among cytokine genes, IL-2 gene is most extensively tried and other cytokine genes frequently examined are IFN-γ, IL-4, GM-CSF and IL-12 [160]. These cytokine genes are used in some cases in combination with the genes encoding costimulatory molecules such as the CD80, which can facilitate antigen recognition process and enhance T cell activation. In preclinical studies, gene transfer of costimulatory molecules increased the susceptibility of gene-modified tumors to host immune system [161]. The other combinatory strategies include simultaneous administration of the gene encoding a putative tumor antigen such as MART-1 in malignant melanomas [162] or an alloantigen such as HLA-B7 [163]. Phase I studies using DNA-conjugated liposome, retrovirus or retrovirus-producing cells showed that the adverse effects caused by gene transfer were minimal and most of the patients are tolerable to the treatment. Limited inflammatory reactions such as pain, swelling and induration at local inoculation sites and transient fever are representative signs and symptoms. Adverse reactions induced by the local administration of adenovirus are similar to those caused by DNA-conjugated liposome and retrovirus. However, intra-arterial administration of adenovirus causes severe liver damages as mentioned. The property that adenovirus tends to infect preferentially hepatocytes, is a benefit to enhance the transduction efficiency of therapeutic

gene(s) into the liver. At present adenoviral vectors are being tested for the treatment of metastatic liver foci via intra-hepatic artery. Adenovirus of higher titer can cause toxic liver damages and in severe cases hemorrhagic tendency is noted. When the liver conditions of the patients are not well maintained, high-dose adenovirus can easily deteriorate the patients' conditions.

Frequency of transgene expression in the patients has been examined. Tissue specimens obtained from the patients are often found to contain the transgene by PCR analysis. Clinical effectiveness of cytokine gene therapy is not fully open to public yet. However, promising results are being reported in several conferences. In melanoma cases, for instance, the recipients developed de novo or increased melanoma-specific, delayed-type hypersensitivity reactions, and $CD4^+$ cell-dominated infiltration around regressing metastases was noticed. None of them exhibited complete or partial regression of main tumors, but some of them experienced a period of disease stabilization, including the shrinkage of a few metastatic foci. A phase II study using liposome-conjugated IL-2 gene showed that more than 50% decrease in the titer of a tumor-associated marker, prostate-specific antigen (PSA), was observed in 38% of the prostate cancer patients, when they received intra-tumoral injection of 300-1500 μg of IL-2 gene [164]. The percentage of the patients whose PSA was decreased more than 25% was over 50%. Since these clinical studies are being examined, the therapeutic potency of cytokine gene therapy is difficult to be evaluated at present. Most of the patients who joined the studies were advanced cases and were refractory to conventional therapies. It also makes the evaluation complicated, because suppression of systemic immune responses is often observed in advanced cases. However, at least in some of the patients, infiltration of immunocompetent cells due to the therapy was detected. This existence of infiltrating cells into the cytokine-producing tumor indicates that immunological responses are actually produced in the patient whose immunity is tolerant to the tumor, although the infiltration may not be directly linked with the clinical outcomes. While the number of responded patients may not be large, these cases are quite important for further analysis on why some patients responded to the therapy and others did not. Investigation on TCR of the infiltrating T cells is a basis to identify the tumor antigens and to develop more sophisticated strategy. Transduction of the TCR genes that are preferentially used in TILs into peripheral blood T cells should potentiate cytolytic activity for tumor elimination [165].

FUTURE DIRECTION

To produce better therapeutic effects, several strategies is to be investigated. The first tactics is to explore the biological functions of DC that have potent antigen processing activity. These cells are capable of potently activating $CD4^+$ T cells, and releasing cytokines such as IL-2, IL-12 and IFN-γ to promote T cell maturation and proliferation. DC may be the only APC that can present a putative tumor antigen via class I molecules to CTLs. CTLs that can recognize the antigen-MHC complex proliferate with the help of $CD4^+$ helper T cells. Therefore, interactions between DC and $CD4^+$ helper T cells, and/or DC and $CD8^+$ CTLs, are crucial for successful generation of anti-tumor response [Fig. (1)]. What kind of signals is important for the activation of DC and acquisition of antigen processing capacity? DC express CD40 molecule on the surface and stimulation of the CD40-mediated signal pathway enhances the ability of DC to process foreign antigens. Stimulation of CD40 molecule on DC is in general mediated by CD40L that is mainly expressed on $CD4^+$ helper T cells. When DC are activated by CD40L using genetically-modified soluble CD40L or by the transfection of CD40 L gene into DC, anti-tumor activity can be enhanced. The other strategy is to express TNF-α gene in DC. TNF-α activates DC in an autocrine or paracrine manner and promotes the antigen presentation activity. Some of previous studies support this notion [166].

Since DC can be semipurified from bone marrow or peripheral blood cells [20], the source of DC cannot be a limiting factor. However, the morphological characteristics of DC and cell surface markers of DC are not consistent. Consequently, it is difficult to identify the authentic DC lineage at present and to define the biological properties. Future studies should solve these problems. Although population of DC is not identical among the studies, several preclinical studies showed that intra-tumoral injection of DC itself can induce adequate anti-tumor responses. Antigen-pulsed DC are more potent to induce systemic immune responses [167]. Since tumor antigens are not well established except in the case of melanoma, the use of antigen-pulsed DC for clinical studies is restricted at present. However, transduction efficiency of DC with an adenoviral

vector is relative high and DC engineered to load a tumor antigen via adenovirus can possibly be one of the strategies for immune-based gene therapy.

Combination of cytokine genes is another direction. In preclinical studies, coexpression of IL-4 and GM-CSF in tumor cells could induce significant anti-tumor activities [101], although the expression of either cytokine was not effective. The combination seems to promote antigen presentation process and is frequently used to purify and differentiate DC in culture. Suicide gene therapy coupled with the expression of cytokine gene is the other combinatory strategy. Expression of herpes simplex virus-thymidine kinase gene in tumor cells followed by administration of a prodrug, ganciclovir, effectively destroys the tumor masses. Tumor cell death by the suicide gene/prodrug system can stimulate host immune responses [168]. The animals that had eliminated tumors by suicide gene therapy become resistant to parental tumor cells that are subsequently inoculated. Although the molecular mechanism of how tumor cell death produces systemic immunity is not fully understood, several cytokines are known to be involved in the immune response [169]. Therefore, combination of suicide gene therapy and cytokine gene therapy using IL-2 and/or GM-CSF is a possible strategy to enhance the therapeutic efficacy [170].

Cytokine gene therapy has benefits over the therapy using recombinant cytokine proteins. At present, the effectiveness of cytokine gene therapy in clinical trials has not been clearly demonstrated. However, the gene therapy in general does not impair the quality of patient's life in contrast to conventional therapies. One of the directions of immune response-based treatment is to identify tumor antigens and to induce CTLs specific for the antigen peptides as mentioned. The strategy is authentic and straightforward. However, identification of tumor antigens requires the establishment of T cell lines. Moreover, tumor antigens can be different, depending on the class I antigens that encompass the tumor peptides. In melanoma, the peptide antigens harbored in class I molecules are known to be different in individuals according to the heterogeneity of class I antigens. If this is true for other tumors, preparation of a set of tumor antigens for each patient having different MHC is not practical. In contrast, expression of cytokine gene can be achieved in every kind of tumors and can potentially induce CTLs that are specific to the tumors. Cytokine gene therapy thereby circumvents the issue of identifying a specific tumor antigen.

Cytokine gene therapy is a good model to analyze the tolerant immune state found in cancer patients. Tumor cells progressively proliferate in spite of immune responses against them. Tumor cells sometimes secrete immunosuppressive cytokines such as IL-10 or TGF-β. Forced expression of IL-2 gene can overcome the immune suppression and then tumor cells can come to regress. One of the reasons that immune responses do not work against tumors is inhibition of TCR-mediated signalings in T cells. In cancer patients, signal transduction within T cells is often impaired. In particular, ζ chains of the CD3 complex in T cells obtained from tumor-bearing animals are not phosphorylated or even disappeared [171,172]. Dephosphorylation state of the ζ chain cannot transmit the activation signals to the nucleus and secretion of IL-2 from T cells does not occur. This tolerant state is restored by local secretion of IL-2 [172]. Therefore, cytokine gene therapy can break the anergy state induced under tumor-bearing conditions. Conversion of immune tolerance to active immune state can be made in cancer patients by manipulating cytokine gene delivery or administering genetically-modified cells.

ABBREVIATIONS

CTLs	=	Cytotoxic T lymphocytes
FasL	=	Fas ligand
TCR	=	T cell receptor
NK	=	Natural killer
MHC	=	Major histocompatibility complex
APC	=	Antigen presenting cells
DC	=	Dendritic cells
CD40L	=	CD40 ligand
TNF	=	Tumor necrosis factor
IL	=	Interleukin
IFN	=	Interferon
GM-CSF	=	Granulocyte macrophage-colony stimulating factor

Th1	=	Type 1 helper T
Th2	=	Type 2 helper T
LAK	=	Lymphokine-activated killer
TILs	=	Tumor-infiltrating lymphocytes
IP-10	=	Interferon-inducible protein 10
Mig	=	Monokine induced by interferon-γ
SCID	=	Severe combined immunodeficient
PSA	=	Prostate-specific antigen

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APPENDIX C

RELATED PROCEEDINGS

(none)